Magnetic Resonance Imaging Determination of Tumor Grade and Early Response to Temozolomide in a Genetically Engineered Mouse Model of Glioma

Patrick McConville,¹ Dolores Hambardzumyan,³ Jonathan B. Moody,¹ Wilbur R. Leopold,¹ Alicia R. Kreger,¹ Michael J. Woolliscroft,¹ Alnawaz Rehemtulla,² Brian D. Ross,² and Eric C. Holland³

Abstract

Purpose: The median survival for patients diagnosed with glioblastoma multiforme, the most common type of brain tumor, is less than 1 year. Animal glioma models that are more predictive of therapeutic response in human patients than traditional models and that are genetically and histologically accurate are an unmet need. The nestin tv-a (Ntv-a) genetically engineered mouse spontaneously develops glioma when infected with ALV-A expressing platelet-derived growth factor, resulting in autocrine platelet-derived growth factor signaling.

Experimental Design: In the Ntv-a genetically engineered mouse model, T2-weighted and T1-weighted, contrast-enhanced magnetic resonance images were correlated with histology, glioma grade (high or low), and survival. Magnetic resonance imaging (MRI) was therefore used to enroll mice with high-grade gliomas into a second study that tested efficacy of the current standard of care for glioma, temozolomide (100 mg/kg qdx5 i.p., n = 13).

Results: The Ntv-a model generated a heterogeneous group of gliomas, some with high-grade growth rate and histologic characteristics and others with characteristics of lower-grade gliomas. We showed that MRI could be used to predict tumor grade and survival. Temozolomide treatment of high-grade tv-a gliomas provided a 14-day growth delay compared with vehicle controls. Diffusion MRI measurement of the apparent diffusion coefficient showed an early decrease in cellularity with temozolomide, similar to that observed in humans.

Conclusions: The use of MRI in the Ntv-a model allows determination of glioma grade and survival prediction, distribution of mice with specific tumor types into preclinical trials, and efficacy determination both by tumor growth and early apparent diffusion coefficient response.

Gliomas are the most common form of brain tumor, with more than half belonging to the most aggressive subtype (i.e., glioblastoma multiforme) at initial diagnosis. Although aggressive combination therapies, including surgery, radiation, and chemotherapy, are commonly used, median survival for patients diagnosed with glioblastoma multiforme is less than 1 year (1). Part of the reason for the deficit in significant progress has been the lack of predictive preclinical models. Development and validation of models that accurately recapitulate the complex-activated signaling pathways involved in glial transformation and tumor progression has been hindered due to lack of available methods for selecting and placing tumored mice on study and for efficient end point determination. However, recent developments in preclinical imaging technologies are facilitating the development of genetically engineered mouse (GEM) models of glioma (2, 3).

RCAS/tv-a technology relies on somatic gene transfer through infection by RCAS viral vectors derived from the avian retrovirus A (ALV-A) in mice expressing the gene for the RCAS receptor (tv-a). The nestin tv-a (Ntv-a) mouse expresses tv-a under the control of the nestin promoter in glial progenitors. The inherent flexibility of this model with respect to choice of the oncogene(s) that induce(s) tumor formation is a key advantage, providing opportunities not only for study of cytotoxic therapies but also new classes of anticancer drugs that target specific oncogenic pathways. When infected with ALV virus encoding platelet-derived growth factor (PDGF), the Ntv-a mice spontaneously develop gliomas by 3 weeks of age in almost 100% of appropriately infected mice, with 30% of these tumors displaying high-grade histologic features (4). Recent results showing the similarity between Ntv-a gliomas and human gliomas (5) suggest that the PDGF-driven tv-a model may be a more predictive model for preclinical evaluation of therapies than the currently available human xenograft or syngeneic rodent models.
This model has been used in several preclinical trials looking at the effects of blockade of PDGF receptor, mammalian target of rapamycin, and Akt (3, 4, 6). Finally, this model has also been used to look at Akt inhibition alone or in combination with temozolomide (6). However, all of these trials were short (7 days), and the effect of these inhibitors and temozolomide over this period was primarily loss of proliferation and not cell kill or apoptosis.

Preclinical evaluation of agents using these models would greatly benefit from the establishment of additional quantitative endpoints for growth and treatment response that could be correlated with survival and translated into clinical trials. Magnetic resonance imaging (MRI) and spectroscopy offer excellent opportunities for noninvasive determination of transgenic tumor induction, and growth and response to treatment dynamically over time in individual animals and cohorts of animals. There are a multitude of quantifiable MR-detectable variables that may provide valuable insights into the pathophysiology and treatment response of the tumor. Among these, proton MR spectroscopy (7–9), perfusion/vascular permeability (10–13), and water-apparent diffusion (14) measurements have shown promise. Perfusion/permeability MRI has been used for brain tumor characterization and for monitoring vascular response to a variety of interventions (15–18). Diffusion MRI allows the movement (Brownian motion) of water within the tumor to be quantified (14). Therapy-induced cell kill is reflected as a net decrease in the restricted movement of water within a tumor, which can be detected as an increase in apparent diffusion coefficient (ADC) values (19). This has been recently reported in a variety of tumor models and more recently in brain tumor clinical studies (19–46). Thus, MRI biomarkers such as these provide opportunities to investigate their sensitivity and specificity in preclinical studies and to subsequently translate them along with promising agents into clinical trials.

In this study, MRI was firstly used to characterize growth and contrast enhancement (vascular permeability) during tv-a glioma tumorigenesis. MRI identified high- and low-grade brain tumors wherein high-grade tumors were distinguished by faster growth rates and were contrast enhancing compared with low-grade tumors that grew more slowly and did not enhance. MRI was then used to enroll mice with high-grade tumors into a preclinical trial using temozolomide. High-grade gliomas were found to be responsive to temozolomide treatment as quantified by a decrease in MRI-detected growth rate and enhancing tissue volumes were derived from these multi-slice T1-weighted images, and a consideration of the total tumor volume, confirmed by histology to correlate with tumor tissue. The enhancing tumor development using MRI. In this study, 35 mice confirmed by MRI to have gliomas at 3 weeks of age underwent weekly T2-weighted and contrast-enhanced T1-weighted (CE) MRI, using multi-slice fast spin-echo and spin-echo sequences, respectively. MRI was done using a Varian Unity Inova MRI system equipped with a Magnex 7T horizontal bore magnet. dMRI was done using a motion-compensated, motion-robust isotropic diffusion sequence using a previously described method (37). Briefly, a trace diffusion weighted multi-slice spin-echo sequence (with motion compensation and a navigator echo) was used to acquire 13 slices with two different diffusion weightings $b = 100 \text{s/mm}^2$ (single acquisition) and $b_2 = 1,248 \text{s/mm}^2$ (two averages). Images were acquired using contiguous transaxial slices with a thickness of 0.75 mm, image matrix 128 $\times$ 128 (zero filled to 256), field of view 20 $\times$ 20 mm, repetition time 2 s, and echo time 60 ms for an acquisition time of 12 min. During all MRI procedures, animals were anaesthetized using isoflurane, and body temperature was maintained at 37°C using a heated re-circulating water pad. T2-weighted images were acquired using a fast spin-echo sequence with a TR/TE = 4,000/60, 8 echoes, echo spacing of 12.5 ms, 17 contiguous transaxial slices, 0.5-mm slice thickness, and an acquisition time of 2 min. T1-weighted images were acquired using a spin echo sequence with TR/TE = 600/20, 11 contiguous slices of 0.5-mm thickness, and an acquisition time of 2.5 min. The average total exam time for each animal was 20 to 25 min.

All tumors were analyzed using a customized volumetric region-of-interest drawing tool in Matlab to segment tumor from normal tissue, with separate regions of interest drawn for the diffusion and T2- and T1-weighted images. Tumor boundaries were manually determined based on hypo-intense regions in T2-weighted images and low–b value diffusion images, with large cystic regions excluded. In this model, T2-weighted hypo-intensities have been previously confirmed by histology to correlate with tumor tissue. The enhancing tumor volume was delineated based on pre- and post-contrast T1-weighted images, and a consideration of the total tumor volume, based on the inherently co-registered T2-weighted images. Tumor and enhancing tissue volumes were derived from these multi-slice MRI data sets over time for each individual animal as previously described (47). At 7.5 weeks of age, half of the mice were sacrificed for histology, with the remaining mice continuing on study for survival analysis.

The second group of 25 mice that were selected as having high-grade gliomas at 4.5 weeks of age were divided into two groups (DMSO vehicle control: $n = 12$ and temozolomide treated: 100 mg/kg qdx5 i.p. beginning on day 1, $n = 13$) with equal distribution of tumor size based upon MRI measurements. Temozolomide was dissolved in DMSO (33 mg/mL) over brief heating and sonication. Baseline dMRI measurements were taken just before treatment (day 0) and again on days 3, 7, 10, and 14 and weekly thereafter. This protocol allowed characterization of tumor growth and ADC response. Survival data were collected under approval from MIRACUC guidelines for oversight, housing, monitoring for condition, and euthanasia requirements.

Histopathology. Animals used for histologic analysis were euthanized, and brains were removed and fixed in 10% neutral buffered formalin for 72 h. Fixed tissues were then transferred to 70% ethanol for 48 h and embedded in paraffin. Formalin-fixed, paraffin-embedded specimens were serially sectioned and slide mounted. The sections were deparaffinized in histo-clear (Richard-Allan Scientific) and were passed through graded alcohols before staining with H&E reagent. The sections were deparaffinized in histo-clear (Richard-Allan Scientific) and were passed through graded alcohols before staining with H&E reagent. For proliferating cell nuclear antigen staining antigen retrieval was done by submerging slides in 0.01 mol/L citric acid (pH 6.0) in a staining jar in a boiling water bath for 30 min. After a 0.3% H2O2 (Sigma) treatment for 15 min, sections were incubated with blocking reagent.

Materials and Methods

Generation of mouse brain tumors. Ntv-a mice were injected i.c. with $10^6$ DF-1 cells transfected with RCAS-PDGF retroviral vectors within 24 h of birth, as previously described (3). Two different groups of animals were injected in the two separate studies described below: the tumor grade study (group 1, $n = 35$) and the temozolomide efficacy study (group 2, $n = 25$). Mice were monitored daily for symptoms of tumor development. From 4 to 5 weeks of age, all animals underwent MRI screening for tumor development. MRI examination of mice. Group 1 was used to characterize glioma development using MRI. In this study, 35 mice confirmed by MRI to have gliomas at 3 weeks of age underwent weekly T2-weighted and contrast-enhanced T1-weighted (CE) MRI, using multi-slice fast spin-echo and spin-echo sequences, respectively. MRI was done using a Varian Unity Inova MRI system equipped with a Magnex 7T horizontal bore magnet. dMRI was done using a motion-compensated, motion-robust isotropic diffusion sequence using a previously described method (37). Briefly, a trace diffusion weighted multi-slice spin-echo sequence (with motion compensation and a navigator echo) was used to acquire 13 slices with two different diffusion weightings $b = 100 \text{s/mm}^2$ (single acquisition) and $b_2 = 1,248 \text{s/mm}^2$ (two averages). Images were acquired using contiguous transaxial slices with a thickness of 0.75 mm, image matrix 128 $\times$ 128 (zero filled to 256), field of view 20 $\times$ 20 mm, repetition time 2 s, and echo time 60 ms for an acquisition time of 12 min. During all MRI procedures, animals were anaesthetized using isoflurane, and body temperature was maintained at 37°C using a heated re-circulating water pad. T2-weighted images were acquired using a fast spin-echo sequence with a TR/TE = 4,000/60, 8 echoes, echo spacing of 12.5 ms, 17 contiguous transaxial slices, 0.5-mm slice thickness, and an acquisition time of 2 min. T1-weighted images were acquired using a spin echo sequence with TR/TE = 600/20, 11 contiguous slices of 0.5-mm thickness, and an acquisition time of 2.5 min. The average total exam time for each animal was 20 to 25 min.

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from MOM kit (Vector Laboratories). Subsequently, sections were incubated with primary antibody anti–proliferating cell nuclear antigen (Oncogene, 0.8 μg/mL) overnight at 4°C. After incubation with secondary biotinylated anti-mouse IgG for 1 h at room temperature, sections were incubated for 30 min with ABC (Vectastain kit; rabbit IgG) then reacted with DAB Detection kit containing Blocker D, Copper D, Inhibitor D, streptavidin-HRP D, DAB D, and DAB-H2O2 D (Ventana Medical Systems) according to the manufacturer instructions.

Results

Imaging characteristics divide gliomas into two groups and predict tumor growth rate and aggressiveness. Intracerebral tumors could be detected using MRI in about 80% of animals at 4 to 5 weeks of age. Tumors were delineated from normal brain tissue as a hyperintense region in T2-weighted images. Tumor volumes were defined for each animal slice by drawing a region of interest encompassing the hyperintense region that was found to correspond well with tumor tissue as identified on histologic sections as shown in Fig. 1. Tumor volumes at 4 to 5 weeks of age were found to range from 5 to 30 μL. Weekly MRI exams revealed two distinct types of tumor profiles as identified by differences in growth rate as well as differences in contrast enhancement and spontaneous large cystic regions. The first tumor type was classified as high grade ( ~ 40% of tumors imaged), and the other tumor type was identified as low grade, which occurred in the remaining 60% of animals (Fig. 1). Table 1 summarizes the characteristics of these two tumor types. High-grade tumors had twice the growth rate compared with low-grade tumors and were typically thrice larger at the initial MRI screening time point (4.5 weeks after RCAS injection). High-grade tumors also consistently showed a more invasive growth pattern and exhibited gadolinium contrast enhancement in T1-weighted images (Fig. 2), whereas low-grade tumors generally did not enhance, except for a small leakage into the ventricular space. The observed contrast enhancement for high-grade tumors was heterogeneous throughout the tumor mass and occurred in about 20% of the tumor volume in early-stage tumors, increasing to about 50% of the tumor volume for late-stage tumors (Fig. 2).

This localized enhancement commonly manifested itself in a ring pattern (Fig. 2). These contrast-enhancing regions often corresponded with localized hypo-intense regions in T2-weighted images (Fig. 3). ADC values for both tumor types were similar (Table 1) at the initial 4- to 5-week imaging time point and slightly higher than the ADC for normal brain, as measured on the contralateral side of the brain (81 × 10⁻⁵ mm²/s). However, whereas ADC values remained constant over

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Low-grade glioma</th>
<th>High-grade glioma</th>
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<tbody>
<tr>
<td>Incidence</td>
<td>22/37</td>
<td>13/37</td>
</tr>
<tr>
<td>Mean tumor size at 4-5 wk (μL)</td>
<td>11 ± 28</td>
<td>31 ± 7</td>
</tr>
<tr>
<td>Mean tumor volume doubling time (d)</td>
<td>20 ± 2</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Mean contrast-enhancing volume at 4-5 wk</td>
<td>—</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Mean ADC at 4-5 wk (10⁻⁵ mm²/s)</td>
<td>84 ± 2</td>
<td>84 ± 2</td>
</tr>
<tr>
<td>Median survival (d)</td>
<td>158</td>
<td>54</td>
</tr>
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</table>

NOTE: Values are expressed as mean ± SE.
time for the high-grade tumor, low-grade tumors were found to have increasing ADC values over time (0.62 ± 0.08 x 10^{-5} mm^2/s/d; Fig. 4). A significant difference was found in survival between mice bearing high- and low-grade tumors (Fig. 4), with median survival of 54 and 158 days, respectively.

**Contrast enhancement of gliomas in mice correlates with histologic features defining glioma grade in humans.** The high- and low-grade tumor classifications were confirmed by histology from animals sacrificed at 7 to 8 weeks. The tumors classified as low grade by imaging criteria uniformly showed diffuse invasion of the normal brain structures by cells with oligodendroglioma-like morphologic characteristics. The tumors classified as high grade by imaging criteria had histologic evidence of pseudopalisading microvascular proliferation, high cell density, and hemorrhage. The T2-weighted MRI hypo-intense regions in these tumors were confirmed by histopathology to correspond with regions of high vascular proliferation and associated high cellular density in six of six cases (Fig. 3). In all six cases, these regions were also associated with contrast enhancement observed in the post-contrast, T1-weighted images. These features were not found in the lower-grade gliomas in this study.

The heterogeneous growth rate seen within the population of GEM gliomas could cause substantial noise in clinical trials with patients harboring these tumors. However, the above data indicate that the presence of contrast enhancement on initial MRI scan predicts gliomas with a relatively uniform aggressive behavior. Therefore, we used the presence of regions with high vascular proliferation in images to stratify tumors and enroll these mice with high-grade gliomas into a preclinical trial using temozolomide treatment.

**Characterization of response of GEM high-grade gliomas to temozolomide.** We mimicked the treatment of human high-grade gliomas by enrolling on trial a cohort of 25 mice identified as harboring high-grade gliomas with abnormal, vascular proliferate regions. The mice were treated with vehicle or a single course of temozolomide (100 mg/kg i.p.) daily for 5 days and then followed by MRI (T2 weighted and diffusion) to noninvasively measure tumor volumes over time and in response to therapy (Fig. 5). The mice tolerated this treatment well, showing a maximum of 12.6% mean body weight loss, 3 days after the end of treatment that was fully recovered 9 days later. Previous data have indicated that this treatment protocol did not result in increased apoptosis within 5 days (6). Consistent with this observation, temozolomide therapy did not result in regression of any of the tumors; however, this treatment resulted in a growth delay of tumor mass of ~14 days followed by a return to pretreatment growth levels at the time of progression. The net cell kill was 0.4 log unit, corresponding to an estimated surviving tumor fraction of 44%.

Diffusion MRI was also evaluated in this study as a potential imaging biomarker for detection of early treatment response because the water-apparent diffusion coefficient is sensitive to changes in tissue cellularity induced by cell kill. The treated group was found to have a significant and early increase in ADC values, which preceded significant deviation in volumetric tumor growth rates between treated mice and controls (Fig. 5). Changes in diffusion values were detectable as early as 4 to 5 days following initiation of treatment. In the treated group, ADC values continued to increase during and for 2 weeks following conclusion of the fractionated dosage schedule.
The subsequent decrease in tumor ADC values at 4 weeks posttreatment correlated with the onset of tumor progression. A significant difference in animal survival was observed between the two groups, with a median survival in the control and treated groups of 52 and 70 days, respectively (Fig. 5).

Discussion

RCAS/tv-a–based GEM glioma models represent an opportunity for studying correlates between histology and imaging characteristics and for developing and testing of new therapeutic strategies for this disease. We characterized tumor development characteristics of PDGF-induced Ntv-a gliomas using a multimodal MRI approach and quantified the response of GEM gliomas to temozolomide, the current standard of care chemotherapy for gliomas in humans.

Infection of Ntv-a mice with RCAS-PDGF viral vectors encoding PDGF resulted in glioma formation in 80% of animals that could be classified as high or low grade using MRI at 4 to 5 weeks of age. High-grade tumors, defined by vascular proliferative regions and contrast enhancement, were characterized by more rapid growth compared with low-grade tumors (Fig. 1; Table 1) and a substantial reduction in the median survival relative to the non-enhancing low-grade tumors (Fig. 4). Tumor contrast enhancement is generally indicative of malformed, leaky microvasculature and high vascular permeability, and has been shown to correlate with histologic grade in human brain tumors (47). In the tv-a tumors, the enhancing volume was localized within the tumor volume as defined by T2 hyper-intensity (Fig. 3), in some cases showing a ring enhancement pattern (Fig. 2). These properties are typical of the contrast-enhancing properties of high-grade human glioma (48, 49), which commonly shows non-uniform, localized appearance that is frequently observed in a ring pattern, presumably due to the presence of necrotic, non-enhancing tissue inside the contrast ring and a periphery of abnormally vascularized, leaky tissue, with associated high vascular permeability. Similar to the human pathology, contrast-enhancing regions were shown by histology to be associated with vascular proliferative, cellular dense tissue (Fig. 3).

Fig. 3. Comparison of T2-weighted MRI (A), T1-weighted MRI, pre-contrast (B) and post-contrast (C), and histologic sections (D and E) of a high-grade glioma, showing vascular proliferation. Four contiguous slices are shown for the MRI. The histologic sections (magnification, ×100) correspond with the region of contrast enhancement in (C) (white arrow). The vascular abnormalities as verified by H&E-stained (D) and CD31-stained (E) histologic sections, appeared as hypo-intense regions on the T2-weighted scans (A) and contrast-enhancing regions in the T1-weighted, post-contrast scans (C). These features were found in all high-grade tumors. Bar, 100 mm (D and E).
In the high-grade tumors, not only did the contrast-enhancing volume increase with tumor growth, but the contrast-enhancing fraction of the tumor also increased from 20% by 4.5 weeks after RCAS injection to >50% by 7.5 weeks after RCAS injection and, in some cases, as high as 60% to 70%. This progression indicates an increase in the fraction of tissue with high vascular permeability or leakiness and implies regional conversion from low to high grade within a given tumor. By comparison, tumors initially identified as low grade (lacking contrast enhancement consistent with low-grade human gliomas; ref. 48) remained so and did not acquire enhancing high-grade characteristics over the period of this study.

Diffusion MRI characterization showed that the ADC increased with tumor growth in low-grade tumors but remained comparatively constant in high-grade tumors. The tumor ADC is sensitive to the average translational water mobility, which depends on the tissue cellularity. Both low- and high-grade tumors generally showed greater ADC compared with normal brain, consistent with a greater extracellular fraction in the tumor tissue, compared with normal tissue. Histologic assessment of low-grade tumors revealed diffuse tumor cells scattered throughout the normal brain tissue. An increasing ADC suggests that low-grade tumor cells were continuing to displace normal brain cells and were becoming...
a larger percentage of the tumor tissue mass over time due to the proliferation of the tumor cells. In contrast, normal brain tissue was completely displaced by the presence of a high-grade tumor, and as such, the mean diffusion values for high-grade tumors showed a small, early increase (Fig. 4C), then remained much more constant. The early ADC increase was presumably due to early displacement of normal cells by tumor cells. The greater ADC of the advanced low-grade tumors compared with the high-grade tumors is consistent with inherently lower cellular density/lower extracellular fraction in the high-grade tumors, compared with the most advanced low-grade tumors.

The ability of MRI to not only determine tumor burden but also to distinguish the vascular and cellular characteristics of high- and low-grade tv-a gliomas suggests that these methods may also be used to determine response to therapy. An initial validation of this hypothesis was done by temozolomide treatment of mice identified as having high-grade tv-a gliomas by MRI characteristics. Treatment with temozolomide resulted in a tumor growth delay of 14 days, compared with vehicle-treated controls. In addition, an early increase in ADC was observed, consistent with decreasing cellularity in response to treatment. A later decrease in ADC correlated with tumor regrowth and was likely due to increasing cellularity with tumor repopulation.

The tnv-a model of glioma provides for a recapitulation of specific biological processes involved in oncogene-specific neoplastic transformation, along with subsequent tumor development and progression. These features constitute a model that is potentially more predictive of the clinical effectiveness of an experimental agent and that may significantly facilitate preclinical testing of new therapeutic strategies, including combination treatments. However, despite the use of a single oncogenic pathway (in this case, the PDGF autocrine loop), GEM gliomas consistently show heterogeneities both between different tumors and within a single tumor, similar to that observed in the human condition. MRI is therefore an important, high-resolution imaging tool for determining tumor grade and type in this model, enrolling subjects into preclinical trials, and for characterizing tumor heterogeneities. The additional use of diffusion MRI allows sensitive detection of early therapeutic effects in tv-a glioma, predicting later gross effects on tumor growth. This highlights the potential for tumor ADC as a surrogate marker for efficacy in human glioma patients.

References
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